Angiogenesis in human liver tumors

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CHAPTER .VI

Summary, General Discussion and future perspectives

Summary

Hepatocellular carcinoma (HCC) is the fifth most common tumor worldwide, and the third cause of cancer-related death.[1] The grave prognosis of HCC is a consequence of the absence of adequate curative options. Angiogenesis is today considered the most important mode of tumor growth associated neovascularization and hence an interesting target for therapy. Insight in the molecular angiogenic features of HCC is pivotal for selection of the proper drug class and treatment regimen tailored for the disease.

The angiogenic status of human liver tumors is not investigated to its full extent. In addition, HCCs can arise in cirrhotic liver but also in non-cirrhotic liver. As the molecular program underlying angiogenic sprouting is subject to microenvironmental control, the question arises whether the angiogenic status in HCC occurring in a cirrhotic liver is different from that in a non-cirrhotic liver. In addition to HCC, there are benign tumors in the liver, including focal nodular hyperplasia (FNH) and hepatic adenoma. FNH accounts for 8% of all primary liver tumors and is the second most common benign liver tumor after hemangioma.[2] Hepatic adenoma is rare, but malignant transformation has been described.[3] Similar to HCC, also for these tumors limited knowledge on the cellular and molecular status underlying tumor growth associated neovascularization is available. In this thesis research, we aimed to study the angiogenic status of these various types of liver tumors. We studied the cellular activation and proliferation status, and the accompanying levels and profiles of genes and proteins that are currently considered to be essential for the initial stages of angiogenesis as well as for vascular maturation.

In **chapter 2**, we analyzed in HCC in cirrhotic and non-cirrhotic livers endothelial cell dynamics (proliferation and apoptosis), microvascular density (MVD), and vessel maturation status (pericytes coverage) as indirect markers of angiogenesis, and correlated these features with tumor vascularization on dynamic contrast enhanced CT and with prognosis. We found that in both conditions endothelial cell proliferation and apoptosis were low in tumor microvessels, and that the majority of tumor vessels were covered by pericytes. MVD was negatively correlated with contrast wash out in the portal venous phase of CT scanning. In HCC in non-cirrhotic livers for which patients were treated by liver resection, a high MVD was associated with a better prognosis. Endothelial cell dynamics in these tumors predominantly demonstrated quiescence and tumor microvessels mainly exhibited characteristics of mature vessels suggesting that angiogenic sprouting is not a hallmark of clinically detectable HCCs.

Based on the observation in chapter 2 that the tumor vasculature presented itself with a mature phenotype, we hypothesized that tumor neovascularization via angiogenic sprouting is not overtly active in HCC. In

chapter 3, we quantified gene and protein expression of members of the VEGF and Angiopoietin (Ang) systems and studied their cellular localization, both in HCC in non-cirrhotic and cirrhotic livers. We employed real-time RT-PCR, Western blot, and immunohistology, and compared the outcome with highly angiogenic human renal cell adenocarcinoma (RCC). Both HCC from non-cirrhotic and cirrhotic livers expressed VEGF-A and its receptors to a similar extent as normal liver. The Ang-1 expression was slightly increased compared to normal liver, while the Angiopoietin receptor Tie-2 was strongly downregulated in the tumor vasculature. Ang-2 expression was downregulated in both types of HCC compared to normal liver. In contrast, in RCC VEGF-A levels were one order of magnitude higher, and (endothelially expressed) Ang-2 was over 30 fold increased compared to expression in normal kidney, while Ang-1 expression was decreased. These data imply that in HCCs, irrespective whether they arise in a non-cirrhotic or cirrhotic liver, the tumor vascularization does not originate from Ang/Tie-2 based vascular destabilization and VEGF driven angiogenic sprouting. However, increased CD31 expression and morphological changes representative of sinusoidal capillarization in tumor associated capillaries indicate that vascular remodeling is taking place in HCC.

Besides malignant liver tumors, liver cells can give rise to benign tumor growth. The data obtained in chapter 3 triggered the question whether these tumors exhibit a similar angiogenic profile.

In **chapter 4** we therefore studied gene expression and protein profiles of VEGF, its receptors VEGFR-1 and 2, and of the Angiopoietins and their receptor Tie-2 in FNH and hepatic adenomas (HCA), of which several subtypes exist.[4] Immunohistology was performed for the cellular localization of the proteins and we also studied the expression pattern of CD34 and alpha smooth muscle actin (αSMA). We compared these profiles with those of normal liver samples. We found an increased expression of both CD34 and αSMA in FNH and HCA, each with its own pattern. We observed no changes in expression of VEGF-A, VEGFR-1, and VEGFR-2 in all types of tumors. In contrast, both FNH and HCA were characterized by increased expression of Ang-1, concurrently with increased Tie-2 in FNH, while in both tumor types Ang-2 levels were similar to those in normal liver samples. The up-regulation of Ang-1 in FNH and HCA along with the various morphological vascular abnormalities which characterized FNH and HCA were similar to the vascular features that have been described in animal models following induction of Ang-1/Tie-2 signaling. Our findings of the absence of Ang-2/Tie-2 overexpression and the unaltered VEGF-A status in HCA and FNH implies that in these benign tumors the VEGF-A/Ang-Tie2 angiogenic pathway is not the major pathway for tumor vascularization. Based on our results we suggested the possibility of an Ang-1/Tie-2 driven vascular remodeling.

The last research chapter is dedicated to COX-2, an inducible

immediate-early gene induced by various stimuli, that can stimulate angiogenesis and is associated with tumor growth, invasion and metastasis.[5,6] Targeting COX-2 or molecules downstream of COX-2 has been proposed as a strategy in the prevention and treatment of solid tumors.[7,8] In this **chapter 5**, we quantitatively examined the expression of COX-2 mRNA and protein in normal livers and in the HCCs using real time RT-PCR and Western blot, respectively, and compared COX-2 expression with the expression of angiogenic factors (VEGF, VEGFR-1, VEGFR-2, Ang-1, Ang-2, and Tie-2). We showed that the COX-2 mRNA levels were lower in both cirrhotic and non-cirrhotic HCC as compared to normal livers and corresponding adjacent non-tumorous tissues. COX-2 mRNA expression in HCC was significantly correlated with angiogenic factors which mainly have a role in vessel stabilization. These results suggest that COX-2 may not exert a prominent role in tumor angiogenesis and progression of HCC.

Altogether, we demonstrated that in HCC originating in cirrhotic and in non-cirrhotic livers, endothelial cells of tumor vessel are predominantly quiescent, with the tumor microvessels exhibiting characteristics of mature vessels. HCCs as well as benign hepatic tumors do not exhibit robust angiogenic sprouting activity driven by VEGF-A and the Angiopoietin/Tie-2 system. Also COX-2 does not seem to play a predominant role in vessel destabilization, a prerequisite for angiogenic sprouting. These observations have important implications for therapeutic decision making regarding anti-angiogenesis based therapy of HCC, as will be discussed below.

General discussion and future perspectives

Problems in therapeutically targeting the tumor vasculature

It is a revolution in the treatment of tumors to therapeutically target the tumor vasculature, which is considered genetically stable and hence less adaptive to pharmacological pressure to induce drug resistance. To various extents, all tumor cells are dependent on a blood supply that delivers oxygen, nutrition and growth factors and takes away waste products. Therefore, therapy aimed to attack the tumor vasculature represents a promising strategy for the treatment of cancer and will most likely exhibit its clinical potential in combination with established standard tumor therapy in the future.[9]

In 2007, ten new drugs with anti-angiogenic activity have been approved by the FDA for the treatment of cancer, and at least 43 other anti-angiogenic drugs were in clinical trials in the United States.[10] However, in both preclinical and clinical settings, the resistance to anti-angiogenic therapy is a major problem. The benefits from anti-angiogenic drugs are at best transitory and are followed by tumor re-growth and progression.[11] The large degree of heterogeneity in endothelial behavior in different tumor microenviornmental conditions is likely to play an important role in this resistance. The presence of

numerous different angiogenic molecules in a tumor results in a system with a variety of redundancy pathways. Additionally, the capacity to employ other mechanisms than VEGF- or FGF-driven sprouting angiogenesis to acquire a blood supply, such as intussusceptive angiogenesis, recruitment of endothelial progenitor cells, vessel co-option, vasculogenic mimicry and lymphangiogenesis, provides a tumor with even more possibilities to evade anti-angiogenic therapy.[12,13] Furthermore, there is no biomarker which can identify the patients who will benefit from a certain anti-angiogenic drug. Below, some of these issues will be addressed in more detail, with special reference to liver tumors.

In vitro and animal models do not mimic the status in human tumors

Until now, the majority of cell biological concepts in tumor growth related angiogenesis have been derived from animal studies. Many of these studies employ injection of tumor cell suspensions in a site that is often chosen to analyze specific parameters of interest. For example, for intravital microscopy to monitor on line neovascular sprout formation and e.g., leakiness of tumor vasculature or effects of drugs thereon, skin-fold window chamber assays are extensively used.[14] For many studies on the anti-tumor effects of anti-angiogenic drugs, s.c. inoculated tumor cell models are utilized as they provide an easy means to analyze the effects of the drugs on tumor outgrowth rate. Furthermore, many pre-clinical tumor models make use of human tumor cell growth in an immune compromised environment, thereby neglecting the contribution of immune cells to the neovascularization process. Although the pre-clinical studies have provided a solid foundation for the understanding of many cellular and molecular concepts underlying tumor driven angiogenesis, there is an urgent need to either confirm these concepts in human tumors, or to challenge or re-define them. Only by this means, the gap between the pre-clinical successes and clinical disappointments of tumor vascular directed therapeutics can be bridged.

Animal tumors at early growth represent a condition of synchronized tumor vascular growth. Patients often present in the clinic with excessive tumor burden that is accompanied by intrinsic variations in tumor cell proliferation status and oxygen demand, and consequent differences in demand for neovascularization as a means to support further tumor growth. As a consequence, tumor samples obtained from patients are both highly variable with regard to the location within the bigger tumor and the time frame between the angiogenic switch and obtaining the biopsies. Patient material is hence per definition heterogeneous, yet the only source to study the pathogenetic concepts derived from animal models and their applicability in human tumors. Since this heterogeneity will be a constant feature in which future therapies will have to be applied, a thorough knowledge of the angiogenic status of human tumors is a prerequiste for future success.

Technological issues about analyzing the angiogenic status of (liver) tumors

The studies described in this thesis address the important issue of the angiogenic status of a malignant liver tumor (HCC), and a number of benign liver tumors in patients. Understanding the molecular make up that drives the tumor vascularization process in these tumors may provide important clues for rational drug development and drug treatment schedules if surgery is not a therapeutic option. It can enrich our knowledge about the "soil" environment of the liver which is a frequent site of metastases from other tumors, especially those from the digestive tract like colorectal carcinomas.

HCC is a hypervascular tumor, and is thought to be highly angiogenic.[15,16] This thesis focused on the angiogenic status of liver tumors (not only HCC). In our studies, we did not find a robust pro-angiogenic status of HCC at 3 levels investigated: endothelial cell dynamics (at the cellular level), vessel maturation (at the vascular level), and VEGF / Angiopoietins / COX2 expression (at the molecular level).

The literature often considers vascularity and angiogenesis to be synonymous. However, several issues can be raised which consider this as an unjustified simplification. Firstly, the resolution of imaging techniques like conventional angiography, computed tomographic angiography and magnetic resonance angiography is insufficient to delineate the microvessels which are the result of angiogenesis.[17] Secondly, upregulation of VEGF expression in HCC is often evaluated in relation to hypervascularity on imaging techniques to find support for the correlation between angiogenesis and vascularity.[18,19] VEGF in itself is not able to induce the angiogenic switch, but requires other growth factors, for instance Ang-2, to exert its action. Thirdly, the most pivotal changes in vascularization of HCC at the histological level is the presence of sinusoidal capillarization and the presence of unpaired arteries, and especially these characteristics did not (sinusoidal capillarization) or weakly (number of unpaired arteries) correlate with contrast enhancement in the arterial phase on dynamic computer tomography.[20] Finally, tumor vessels in HCC are characterized by abnormal arterial-venous connections; especially arterioportal and arteriovenous shunts are often identified.[21,22] These shunts, although characterized by large supplying vessels (hypervascularization), represent inefficient tumor angiogenesis because they "bypass" the tumor cells and thereby prevent exchange of oxygen and nutrients. Taken together, these radiological techniques can thus be regarded as reasonable markers of tumor blood flow but at most as indirect, surrogate, and thus unreliable markers of angiogenesis. Future new technical developments will hopefully more clearly delineate which of the many variables of dynamic imaging are most suited for reliable measurement of tumor angiogenesis.[23]

Also, in the benign primary hepatic tumors the studied angiogenic growth

factors imply that VEGF-A driven angiogenic sprouting is not happening in the tumors, and that remodeling, possibly through Ang-1/Tie-2 signaling, is stimulated. From this one can conclude that the vascular remodeling, guided by an altered vascular and sinusoidal milieu, could properly meet the requirements of the tumor's growth and nutritional needs without robust neovessel formation.

There is no extensive solid proof nor consensus to support the concept that HCC is highly angiogenic. Our data on low VEGF expression, the most extensively studied angio-gene in HCC, corroborate a few previously published studies reporting on the absence of upregulation of VEGF gene in HCC.[24] On the other hand, ample studies reported the overexpression of VEGF in human HCC tissues by immunohistochemistry,[19,25,26] which supported the notion that HCC is a highly angiogenic tumor. According to our experience, one should carefully interpret the expression of proteins using immunohistochemistry. As hepatocytes are a rich source of various proteins, it is easy to get non-specific staining. In our research, we found different patterns of VEGF-A, Ang-2, Tie-2 staining between immunostaining on paraffin and on frozen tissues. Immunostaining on paraffin sections showed that VEGF-A is abundant and diffusedly distributed in the cytoplasm of almost all hepatocytes and tumor cells, just like other studies have reported before.[27] In contrast, using the frozen sections of the same cases, the VEGF-A expression was much less abundant and located mainly in the endothelial cells. As a growth factor, VEGF-A was not expected to be abundantly expressed like structural proteins. Moreover, the pattern of VEGF-A expression on frozen sections is more reasonable, and can be explained from the localization of VEGF-A in close proximity with its receptors on endothelial cells. The same observation was done with Ang-2 and Tie-2. Ang-2 expression is mainly restricted to endothelial cells. Immunostaining of Ang-2 on paraffin sections did not show this vascular restriction, while the immunostaining of frozen sections did show that Ang-2 is indeed localized mainly in endothelial cells. Interestingly, in tissue adjacent to tumor or histological normal liver tissues a zonal distribution of Ang-2 in the center of liver lobule could be observed, which can be explained by the relative anoxic environment in the perivenular areas.

Phosphorylation of growth factor receptors and phospho-kinase activity analyses in situ may provide functional information on the activity of angiogenic factors. At present, however, the technology of anti-phospho-protein immunodetection is not extensively applied, mainly because of lack of special antibodies to phosphorylated proteins. Moreover, the existence of various phosphorylation sites in a single receptor or kinase protein can make interpretation of data rather cumbersome. Our data suggests that the hepatic tumors do not have an overt manifestation of angiogenic activity according to the current model of angiogenic sprouting. In that model, Ang-2 acts as the dynamic factor that is overexpressed and

competes with Ang-1 for Tie-2 binding. As a consequence, Tie-2 is de-phosphorylated to destabilize the vasculature and sensitize the endothelium to VEGF induced proliferation. To further support the existence (or absence) of such a concept in human tumors, it is instrumental to create methods to study the phospho-Tie-2 status in tissue biopsies. Western Blot analysis of phospho-protein in whole tumor extracts or in Tie-2 immunoprecipitated purified protein extracts is feasible nowadays. Yet, details on localization of its presence would provide important added value when considering the tumor and tumor vascular heterogeneity issue. Similarly, immunohistochemical detection of phospho-VEGFR-2 and other kinases proposed to be active during the angiogenic process would create new opportunities to zoom in on the molecular pathology of tumor associated neovascularization processes in human tumors.

Other proangiogenesis factors in liver tumors

The molecular repertoire that tumor cells can use to regulate angiogenesis is diverse and may alter for a given tumor type. Although VEGF has proven to be the most critical angiogenic factor identified to date, there are other pro-angiogenic factors, eg. FGFs, PDGF and EGF.[28-31] Most human cancers can express several proangiogenic proteins.[32-34] Placental growth factor (PlGF), a member of the VEGF family may primarily mediate arteriogenesis, the formation of collaterals from preexisting arterioles, but has also been associated with angiogenesis and was proposed as a potential therapeutic anti-angiogenic target.[35] PDGF-BB and bFGF were recently described to have an important synergistic role in tumor neovascularization and metastasis, without any involvement of VEGF.[36] So in the future, it is worthwhile to investigate other pro-angiogenic factors as potential mediators of vascular remodeling in HCC.

Other mechanisms of tumor vascularization in liver tumors

In recent years, additional mechanisms of tumor vascularization have been recognized, including recruitment of endothelial progenitor cells, vessel co-option, vasculogenic mimicry vessels and intussusceptive vascular growth.[13,37] Vasculogenic mimicry was furthermore recently reported to be present in hepatocellular carcinoma and shown to be associated with tumor recurrence after orthotopic liver transplantation.[38] And, like we pointed out in chapter 1, there is some proof to support that the tumor cells in liver (primary or metastatic tumors) may exploit pre-existing vessels for their growth.

At present, we lack a vascular marker for (sinusoidal) endothelial co-option, and hence cannot test the hypothesis that HCC is nourished by vessels that are coopted instead of being created by angiogenic sprouting. Yet, the finding that HCC can grow in the absence of increased expression of VEGF and a destabilized Ang/Tie-2 phenotype puts forward an intriguing premise that the

permissive role of SEC may be a general feature for tumor growth in the liver. The recent observations that the expression of VEGF-A in primary colorectal carcinoma is higher than in colorectal carcinoma metastases in the liver[39] and that primary pancreatic cancer expresses twice as much VEGF as compared to its metastasis in the liver[40] are compelling indications that the liver niche indeed represents a microenvironment that can actively influence tumor growth characteristics. At present, however, nothing is known about how co-opted blood vessels differ from the normal vasculature and the possible implication for responsiveness/adaptation to anti-angiogenic therapy.[41]

Alterations of sinusoidal endothelial cell phenotype in various liver lesions

The liver is a special organ, receiving 15-20% of the cardiac output, in which approximately one-third from the hepatic artery (well oxygenated blood) and two-thirds from the portal vein (nutrient-rich blood from the digestive tract). The hepatic artery and portal vein both drain into the hepatic sinusoids, which represent the capillary network in the liver.[42] Regenerative and dysplastic nodules in liver have a blood supply predominant from the portal vein, and lack contrast uptake in the arterial phase in contrast-enhanced liver imaging. Upon dysplastic nodules transition into overt HCC, the vessel pattern sharply changes and arterial supply is predominant.[43] From early to fully malignant nodular lesions in liver, the number of unpaired arteries increases. The unpaired arteries refer to arteries not accompanied by bile ducts as in portal tracts of normal liver, and can be highlighted with CD34 and αSMA immunostaining.[44] Scanning electron microscopic imaging shows tumor vessels with irregular diameters and abnormal branching pattern.[45]

Hepatic sinusoidal endothelial cells (SECs) are discontinuous and possess fenestrations, and also display gaps and lack an organized basement membrane.[46] During liver organogenesis, an early structural differentiation of sinusoids, occurring from 5 to 12 weeks of gestation, was characterized by the loss of continuous endothelial cell markers (CD-31, CD34) and a reduction in the peri-sinusoidal amount of laminin and in the deposition of tenascin, fibronectin, and thrombospondin. A later functional differentiation was characterized by the acquisition of the markers of adult sinusoidal endothelial cells (CD4, ICAM-1, CD32, and CD14), during 10-20 weeks of gestation. The typical ultrastructures of SECs, including cytoplasmic fenestrations, are not acquired before 17 gestational weeks.[47]

FNH presented a sinusoidal pattern of endothelial cell differentiation. All HCC presented a continuous pattern of endothelial cell differentiation. In hepatocellular adenomas, the intra-tumoral vascular phenotype was heterogeneous, with 50% presenting a sinusoidal pattern, 19.5% presenting a continuous pattern and 30.5% showing a mixed pattern.[48]

As pointed by Frachon S et al., there are two possible mechanisms to

explain the alterations of the vascular phenotype. One hypothesis states that tumor-associated capillary vessels are neo-vessels produced through a process of tumor angiogenesis. Another hypothesis is that the phenotypic changes indicate an abnormal differentiation of pre-existing sinusoids.[49] In other words, increasing CD34 expression by endothelial cells might represent a bystander effect of changing influences from adjacent cells or growth factors. The liver sinusoids may represent a specialized vascular bed that preferentially facilitates pathology related demands by vascular remodeling via other mechanisms than angiogenic sprouting. We have observed that in the border of HCC with adjacent liver, the adjacent non-tumorous liver parenchyma shows a very faint staining of CD34 with a gradual increase in staining intensity towards the tumor (see chapter 2). Moreover, CD34 staining is not uniform as both CD34 positive and CD34 negative segments of vessels can be encountered especially in the periphery of HCC. Today, we cannot test those hypotheses. However, investigations of isolated endothelial cells or tumor vessels on the aspect of angiogenic associated factors will be helpful to elucidate microenvironments in which endothelial cells live, and maybe provide a potential target for therapeutically targeting tumor vasculatures in HCC.

Isolation of intra-tumor endothelial cells for detailed angiogenesis studies

To obtain a full angiogenic profile of tumor endothelial cells it is essential to purify the cells from the tumor tissue, as the intra-tumor endothelial cells represent only a small fraction of cells in tumor tissue. Results of analysis of whole tissue samples are usually determined by the major cell type and may mask biologically relevant and important changes present in endothelium. For this purpose, St. Croix et al. isolated endothelial cells by enzymatic digestion of tumor tissue, followed by sorting with magnetic beads. They next used SAGE (serial analysis of gene expression) to compare gene expression patterns of endothelial cells isolated from the blood vessel of normal resting tissues, tumors, and regenerating liver. They found striking differences between physiological and pathological angiogenesis, and confirmed their findings by real time RT-PCR, in situ hybridization, immunostaining and immunoblotting.[50] Isolation of endothelial cells by enzymatic digestion and sorting by magnetic beads, influence endothelial cell behavior in various ways.[12] In addition, one looses information on the original location of the endothelial cells in the (tumor) tissue, and hence information that may be of essential importance in the light of tumor vascular heterogeneity.

The development of laser-based tissue microdissection systems now allows for rapid acquisition of specific morphologically and/or phenotypically distinct types of cells for a number of molecular analyses.[51] In cancer research, laser microdissection has been most extensively used to get tumor cells for subsequent analysis. The endothelial cells are small, and difficult to identify

from other cells in histologically stained tissue. Combined with immunostaining for endothelial or angiogenic markers, laser capture microdissection can be used to get endothelial cells from tissues. Immunohistochemistry-guided LCM has the advantage of capturing ECs from their natural (patho) physiological microenvironment with maintenance of information on localization and associated pathology. Using immuno- laser capture microdissection to get endothelial cells and subsequent genome-wide transcriptional profiling, Buckanovich et al. identified genes that were differentially expressed between vascular cells from human epithelial ovarian cancer and healthy ovaries.[52] Similarly, Bhati et al performed rapid immunohistochemistry using anti-factor VIII-related antibodies on sections of frozen human luminal-A breast tumors and normal breast, followed by laser capture microdissection of vascular cells. They identified 1176 genes that were differentially expressed between tumor and normal vascular cells, 55 had a greater than fourfold increased expression level.[53]

To further detail on the molecular status of the tumor vasculature in liver tumors, the immuno- laser capture microdissection technique is highly suited as a method to isolate endothelium from HCC, adjacent non-tumor tissues, cirrhotic liver and benign liver tumors, and will be pursued in our laboratory in the coming years. Whether the read-out will be quantitative RT-PCR, which represents a biased approach that analyses pre-destined sets of genes, or a genome wide microarray study, will depend on the yield of RNA from the dissected samples and the advances in technologies to amplify the RNA without disturbing the high abundant / low abundant gene profiles of the RNA. Using this approach for HCC and their healthy control tissues, we hope to be able in the future to identify molecular markers that can assist in further unraveling of the actual status of tumor vasculature, for use in daily pathological practice and beyond.

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